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AUTHOR(S):

Kurachi, Mamoru

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Studies on the Biosynthesis of Pyocyanine. (II)

Isolation and Determination of Pyocyanine

Mamoru KURACHI*

(Katagiri Laboratory)

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In the present work, a method for determination of pyocyanine has been established by photometric studies using Beckman model DU spectrophotometer. On the other hand, a colorimetric method in comparison with standard alkaline solution of CuSO_4 has been proposed. Although absorption spectra of the blue pigment regarded as pyocyanine did not wholly correspond to those reported by other workers, this pigment has ultimately been confirmed by its elementary analysis to be identical with pyocyanine itself.

The colorimetric method using standard solution was found to be beneficial not only to simplify the experimental operation but also to use such a standard solution as could be preserved for a long period without any deterioration, although the accuracy was somewhat inferior to the photometric determination using Beckman photometer. In the present paper, absorption spectrum of pyocyanine will rightly be presented so as to be applied as a method for identification of pyocyanine.

INTRODUCTION

Since a method for determination of pyocyanine has not yet been established, the estimation of pyocyanine in the previous work was carried out by the temporary method which expressed merely a relative amount of pyocyanine in comparison with standard solution of CuSO_4 .¹⁾ Hitherto, the study on the determination of pyocyanine seemed to have been disregarded from the ordinary viewpoint on the coloring matter. However, the author has experienced in the present work that precise measurement of pyocyanine was not such an easy matter as had been expected, because of its labile property or of difficulty of its purification.

Pyocyanine was obtained in crystalline form as free base and various derivatives. Among these crystals, only pyocyanine chloroplatinate was successfully obtained in pure state, although each compound could easily be crystallized. Molecular extinction coefficient has been presented previously by the author.²⁾

It has been noted that free form of pyocyanine had never been obtained in pure state by merely repeating recrystallization, although crystals could easily be formed from optional solvent as very fine needles. In the present paper, method of isolation, identification and determination of pyocyanine will be presented in detail.

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EXPERIMENTAL AND DISCUSSION

Isolation and Purification of Pyocyanine

Cultured solution of bacterium *Pseudomonas aeruginosa* was extracted with chloroform to isolate pyocyanine when sufficient pigmentation has taken place. In order to attain complete extraction an emulsion which is usually formed on shaking with chloroform, must be eliminated by centrifugal treatment or by the addition of anhydrous sodium sulfate to the warm chloroform extract. And in order to remove impurities, the chloroform solution of crude pyocyanine was shaken with dilute HCl and the HCl solution of pyocyanine was again extracted with chloroform after being made alkaline with Na_2HPO_4 solution. The same procedures were repeated several times. Since pyocyanine easily breaks down into α -hydroxyphenazine in chloroform solution, the solvent chloroform must be evaporated in vacuum at lower temperature as soon as possible after being dehydrated with anhydrous sodium sulfate. Blue black pyocyanine crystals were formed by the addition of excess of ether or petroleum ether to the concentrated chloroform solution. Although very fine needles could be formed by recrystallization from water and methanol, it always failed to obtain the pure pyocyanine crystals agreeing with the calculated values in elementary analysis. However, it was found that when pyocyanine chloroplatinate was used for the purification of pyocyanine, pure pyocyanine crystals were successfully obtained. And when the crystals were analyzed immediately after the preparation, it was found, C 74.17, H 4.61%; calcd. for $\text{C}_{13}\text{H}_{10}\text{N}_2\text{O}$ (210.09), C 74.27, H 4.49%. (m.p. 133° uncorr.) For the reason why pyocyanine could never be obtained in pure state, although it was easily crystallized as fine needles and yet its crystallization was repeated so many times, it is suggested that even in the crystalline state, there exists some impurity revealing the same properties as those of pyocyanine and that pyocyanine itself is so unstable as to be decomposed into α -hydroxyphenazine. In order to prevent pyocyanine from decomposition, various efforts were made. As the decomposed product of pyocyanine, α -hydroxyphenazine was hardly recognized from the crystals for several days, by extracting with ether, the following procedure was considered to bring some effects: pyocyanine crystals were preserved in brown colored desiccator on P_2O_5 at reduced pressure, or under CO_2 or H_2 gas. However the effect could not be expected for a long time by the above procedure. It is assumed that pyocyanine is slightly basic and its redoxpotential may be kept relatively toward negative, so that pyocyanine is oxidatively demethylated into α -hydroxyphenazine even in the absence of oxygen, as in the case of its alkaline aqueous solution. Since pyocyanine is unstable in chloroform or acetone, the crystals formed from these solvents are desired to be repeatedly washed with ether. In fact, it was observed that the crystals formed from water were more stable than those from other organic solvents. On the other hand, one should be careful for the fact that free pyocyanine crystals absorb HCl gas in atmosphere to form its salt, or that pyocyanine hydrochloride and other acid salts absorb ammonia gas. However, it is observed that pyocyanine is consider-

ably stable in acid aqueous solution as compared with its crystalline form. Hence, it is preferable for the preservation of pyocyanine for a short period to keep it in acid aqueous solution, although pyocyanine chloroplatinate is the most desirable form for keeping for a long period, as will be mentioned in the following section.

Pyocyanine Chloroplatinate and Other Salts

In the present study, the following salts were obtained: pyocyanine hydrochloride, sulfate, perchlorate, chloroplatinate, picrate and oxalate. It was noted that organic acids other than oxalic acid, for example, formic, acetic, tartaric and succinic acids did not form the salt of pyocyanine. Pyocyanine hydrochloride was prepared by the following procedure: crystal of free form was dissolved in methanol and HCl was added drop by drop to it. Reddish soft crystals were immediately formed by the addition of ether to its acid methanol solution and the crystal was washed with the ether saturated with water, and then repeatedly washed with dried ether. Reddish brown crystals were reformed by dropping of ether to its absolute alcohol solution. Pyocyanine chloroplatinate was simply obtained by the addition of chloroplatinic acid to aqueous solution of the hydrochloride. The result of elementary analysis was as follows: Found, C 37.73, H 2.69, Pt 23.85%; Calcd. for $(C_{13}H_{10}N_2O)_2H_2PtCl_6(830.16)$, C 37.85, H 2.67, Pt 23.51%. In spite of the labile property not only of free base but also of many other derivatives pyocyanine chloroplatinate was alone found out to be so stable as to be preserved for several years without any deterioration.

α -Hydroxyphenazine

In the cultured solution of the bacteria, besides pyocyanine, some amounts of yellow pigment, α -hydroxyphenazine ($C_{12}H_8N_2O$) and other green fluorescent pigment which is insoluble in any organic solvents are usually detected. α -Hydroxyphenazine is derived from pyocyanine by its destruction, and this substance somewhat resembles pyocyanine in its behavior toward organic solvents, so that on the extraction of cultured solution, this may ordinarily be accompanied with pyocyanine fraction. However their distribution coefficients between organic solvent and water are different from each other according to the pH of their solution. α -Hydroxyphenazine is recognized in the aged cultured solution and is obtained by heating pyocyanine in alkaline solution. Acidified aqueous solution of α -hydroxyphenazine was extracted with chloroform or ether and the chloroform extract was shaken with dilute alkali (changes in color to reddish violet) and then this solution was repeatedly extracted with organic solvent after being made acidic. Remained oily substance after evaporating the organic solvent was dissolved in a small amount of methanol or ethanol and then water was added drop by drop to it. The yellow needles formed were further purified by sublimation (m.p. 157° uncorr.).

Absorption Spectrum of Pyocyanine and α -Hydroxyphenazine

Since pyocyanine has hitherto been studied as a biological pigment or an

antibiotic substance, several reports on the spectroscopic study have already been published. Babes³⁾ early stated that absorption spectra of pyocyanine were shown by two bands: with alkaline solution, in the region of 330-660 $m\mu$ and 150-ultraviolet, and with acid solution, ultrared-2600 $m\mu$ and 400-ultraviolet.

According to Cluzet *et al.*⁴⁾, absorption spectrum in ultraviolet region is shown in the region from 358 to 388 $m\mu$ and the maximum exists at 377 $m\mu$.

In a more recent investigation, Ehrismann *et al.*⁵⁾ presented that pyocyanine in alkaline aqueous solution exhibited absorption bands with maxima at 660 and 326 $m\mu$ and with minima at 460 and 281 $m\mu$; in acid aqueous solution, it revealed the bands with maxima at 520, 366 and 281 $m\mu$ and minima at 660 and 326 $m\mu$.

In the present experiment using Beckman spectrophotometer, absorption bands are observed as follows: with neutral or alkaline aqueous solution, maxima exist at 690, 379, 311 and 238 $m\mu$ and minima, at 448, 344 and 270 $m\mu$; with acid aqueous solution, maxima at 520, 387, 278 and 242 $m\mu$ and minima at 422, 318, 251 and 232 $m\mu$ (see Fig. 1).

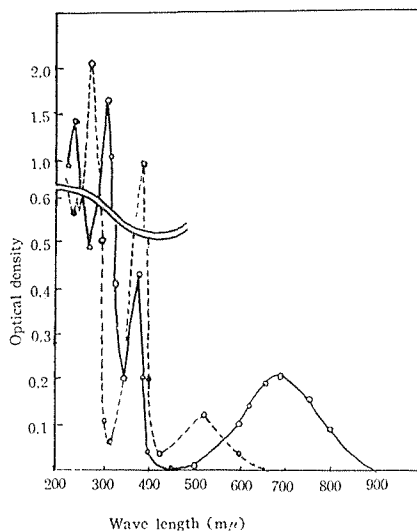


Fig. 1. Absorption spectra of pyocyanine.
(○—○—○) With neutral or alkaline solution, (○·····○) with acid solution. Concentration is approximately $5 \times 10^{-5} M$.

It is noted that although the shape of absorption curves reported by Ehrismann *et al.*, nearly corresponds with the present result, absorption spectra stated by the other workers are considerably different from those of the present experiment.

a-Hydroxyphenazine is soluble in various organic solvent but hardly soluble in water, although it is extremely soluble in alkali, so that its neutral aqueous solution was prepared by neutralizing the dilute alkaline solution. As shown in Figs. 2 and 3, any maximum absorption in visible region is not observed with acid or neutral solution, whereas with alkaline solution, an absorption maximum is revealed at 510 $m\mu$. In ultraviolet region, absorption maxima are shown at 350 and 260 $m\mu$ in each case.

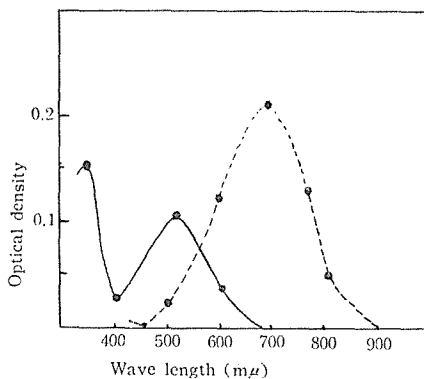


Fig. 2. Absorption spectrum of α -hydroxyphenazine.

(●—●—●) α -Hydroxyphenazine in alkaline solution, (○·····○) pyocyanine in alkaline solution.

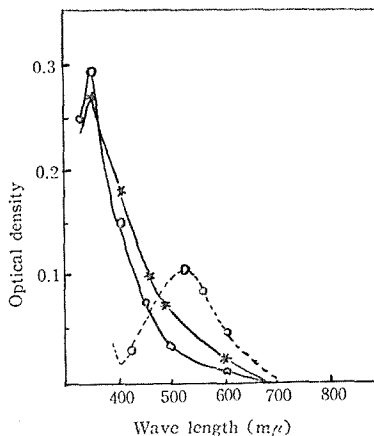


Fig. 3. Absorption spectrum of α -hydroxyphenazine.

(*·····*) With neutral solution, (○—○—○) with acid solution, (○·····○) pyocyanine in acid solution.

Although maximum is not seen with acid or neutral solution, a considerable absorption band is still revealed around 520 $m\mu$ at which pyocyanine exhibits the absorption maximum in acid aqueous solution. In practice, however, such an absorption as above may be negligible, since the amount of α -hydroxyphenazine in the medium is far small as compared with pyocyanine. From these results, it may be concluded that the effect of contamination with α -hydroxyphenazine on the determination of pyocyanine can be neglected, although some effect may be necessary to be taken into account according to the condition of the cultured solution, especially when the measurement is performed with acid solution of the aged cultures.

Photometric Determination of Pyocyanine using Beckman Spectrophotometer

As was already mentioned, free form of pyocyanine was so labile as to be hardly successful in obtaining pure crystal, unless it was immediately supplied to analysis after the preparation, so that pyocyanine chloroplatinate was used for this experiment. Since the crystal of chloroplatinate was hardly soluble in water and other organic solvents but soluble in very dilute NH_4OH solution in which any decomposition of pyocyanine was never found even in the solution of high pH values.

In this case, chloroplatinic acid is separated from pyocyanine to form its ammonium salt, but it was found not to reveal any effect on the result of the determination in visible region. The sample used for the determination was prepared as follows: the entirely dried pure crystal of pyocyanine chloroplatinate was weighed by a micro-balance, dissolved in water made slightly alkaline with NH_4OH and adjusted to the desired volume. According to the direction of Wrede and Strack,⁶⁾ pyocyanine has bimolecular structure, while its leuco-form

shows monomolecular structure. On the contrary, Kuhn and Schön⁷⁾ believed that pyocyanine is constituted with one molecule of phenazine nucleus, and Michaelis *et al.*^{8,9)} confirmed by its potentiometric study that pyocyanine has monomolecular structure at least in its solutional state. At present, the monomolecular structure has generally been recognized, so that molecular extinction coefficient of pyocyanine which had previously been reported, was also expressed as monomolecular structure.

However, the author has come to believe in the course of this experiment that pyocyanine would rather be composed of two molecules of phenazine nucleus in its crystalline state, because the crystals regarded as pyocyanine monohydrochloride which could not be identified by the monomolecular structure, were really obtained.

Regression line. Optical densities were measured at 690 m μ with neutral or alkaline aqueous solution, or at 520 m μ with acid aqueous solution, respectively. In the range of about $2 \times 10^{-4} \sim 10^{-5}M$, the relation between the value of optical density and concentration of pyocyanine was found to be linear according to

Table 1. Analytical data on measurement of pyocyanine.

At 690m μ		At 520m μ	
$S(x)=11.0$	$n=10$	$S(x)=11.0$	$n=10$
$S(y)=2.264$	$\bar{x}=1.10$	$S(y)=1.284$	$\bar{x}=1.10$
$S(xy)=3.1656$	$\bar{x}=0.2264$	$S(xy)=1.7972$	$\bar{y}=0.1284$
$S(x^2)=15.40$		$S(x^2)=15.40$	
$S(y^2)=0.649844$		$S(y^2)=0.209948$	

n =experimental No.

S =the sum.

x =amount of pyocyanine expressed in mg%.

y =value of optical density.

\bar{x} , \bar{y} =arithmetic means.

Beer's law. From the results summarized in Table 1, this relation can be shown by the following equation derived by application of least squares :

$$Y = \bar{y} + b(x - \bar{x})$$

where x is the amount of pyocyanine ; y , the value of optical density ; \bar{x} and \bar{y} are mean values of x and y , respectively. Y is the expected value of y , b is the regression coefficient which is given as

$$\frac{n \sum xy - \sum x \cdot \sum y}{n \sum x^2 - (\sum x)^2}$$

where n is the experimental number. From the data shown in Table 1, the following equations were obtained :

$$Y = 0.00137 + 0.2047x \text{ (at 690 m}\mu \text{ with pyocyanine aqueous solution),}$$

$$\text{or } Y = 0.00014 + 0.1167x \text{ (at 520 m}\mu \text{ with pyocyanine acid aqueous solution),}$$

where x is the amount of pyocyanine expressed in mg% and Y is the expected value of optical density.

The statistic analysis was attempted on the results obtained above and it

was demonstrated by a significance test that there was no significant difference between 0.00137 of the section and zero of the origin (level of significance is 5%; degree of freedom, 8). Therefore, the above regression line may be fixed by the origin to be expressed as the following equation:

$$Y=0.2047 x, \text{ or } Y=0.1167 x \text{ (see Fig. 4).}$$

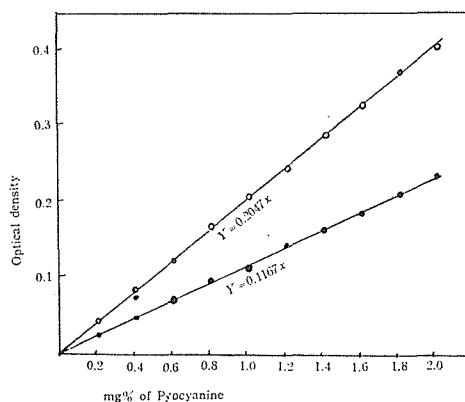


Fig. 4. Relation between optical density and amount of pyocyanine.

(○—○—○) At 690 mμ with neutral or alkaline aqueous solution,

(●—●—●) at 520 mμ with acid aqueous solution.

It is necessary in this case to keep the errors of x smaller in comparison with those of y , so that ordinate was represented by the value of optical density and abscissa, by the amount of pyocyanine. Provided, on the contrary, the amount of pyocyanine was expressed along the ordinate, the regression equation would be given by

$$Y=4.918 x, \text{ at } 690 \text{ m}\mu; \text{ or } Y=8.536 x, \text{ at } 520 \text{ m}\mu.$$

Accuracy of equation. Accuracy of the results of determination by the above equation was tested. As shown in Table 2, the errors were observed to be less than $\pm 3\%$ in the range shown in the table.

Determination of pyocyanine in cultured solution. For the determination

Table 2. Accuracy of estimated values of pyocyanine.

Pyocyanine known (mg%)	No. of measurement	Standard errors		Limit of errors (%)	
		Optical density	Pyocyanine (mg%)		
At 690 mμ	0.5	8	0.102±0.0026	0.5±0.0122	±2.44
	1.0	8	0.205±0.0041	1.0±0.0200	±2.00
	2.0	8	0.409±0.0084	2.0±0.0410	±2.05
	3.0	8	0.614±0.0170	3.0±0.0835	±2.78
At 520 mμ	0.5	8	0.058±0.0018	0.5±0.0153	±3.06
	1.0	8	0.117±0.0032	1.0±0.0274	±2.74
	2.0	8	0.233±0.0062	2.0±0.0530	±2.65
	3.0	8	0.350±0.0088	3.0±0.0751	±2.50

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of pyocyanine in the cultured solution, experiments were carried out by the following procedure: 5 ml of the cultured solution was completely extracted with chloroform and pyocyanine in the chloroform solution was transferred into acid aqueous solution. This red aqueous solution was neutralized (or used without neutralizing for the measurement at 520 $m\mu$) with NH_4OH solution and adjusted to 50 ml. In parallel experiment, the following procedure was attempted to test the effect of other impurity existing in the medium: to 5 ml of the cultured solution taken into another test tube, $ZnSO_4$ solution was added and made alkaline with dilute NH_4OH to precipitate the bacterial cells and other protein substance. The precipitate of the cultured solution was washed down through a filter to obtain blue clear solution, and this solution was adjusted to 50 ml and used for the parallel determination to compare with the sample obtained by extraction treatment. It is obvious from the result shown in Table 3 that under the ordinary condi-

Table 3. Effect of α -hydroxyphenazine on determination of pyocyanine.

Sample	At 690 $m\mu$		At 520 $m\mu$	
	Optical density	Pyocyanine (mg%)	Optical density	Pyocyanine (mg%)
No. 1	0.424	0.207	0.240	0.206
No. 2	0.428	0.209	0.245	0.210
No. 3	0.430	0.210	0.344	0.295

No. 1, cultured solution was measured through the extraction treatment.

No. 2, do. was directly measured without extraction treatment.

No. 3, No. 1 + α -hydroxyphenazine (about 5×10^{-5}).

tion, the contamination due to impurity such as α -hydroxyphenazine and others hardly influences the result of the determination of pyocyanine in cultured solution. Therefore, purification procedure of the sample will not be so much required, if the solution is completely clear. Although even with the acidified solution of the cultures, a noticeable effect of impurity will not be seen in the above-mentioned experiment, it may, in general, be desirable for the determination of pyocyanine in cultured solution, especially in the aged cultures with which extraction procedure is omitted that the measurement is performed at 690 $m\mu$ with neutral or alkaline solution. On the other hand, one should be careful for the decrease in the amount of pyocyanine based on its decomposition by heating or making alkaline on the way of preparation of the sample, and for the fact that at pH of about 5, pyocyanine solution changes in color to violet and optical density becomes only half value of the normal state of blue or red colored solution.

Colorimetric Determination in Comparison with Standard Solution of $CuSO_4$

The photometric method using Beckman spectrophotometer is, of course, very sensitive and precise result may be expected. However in practical measurement in the experiment of this series, it is not unusual to compare pyocyanine to extent over several times concentration. Therefore, it may not be useless to

attempt similar method of the determination as long as the experimental operation is simplified, although the accuracy is somewhat inferior.

In the colorimetric method, standard solution is, in the first place, required to correspond in color tone to the sample, so that it is desirable for the standard solution to use the substance itself to be determined, if it is possible. However, as was often mentioned, pyocyanine is so unstable as to be hardly preserved, so that a substitutional standard solution was obliged to be chosen. For the standard solution, CuSO_4 brings about the objection that its color tone is not fully agreeable with pyocyanine. As an innovation the following experiment has been attempted. It is a well known fact that in the presence of tartrate, salt of heavy metals such as Cu or Fe can be protected from the precipitation even in its stronger alkaline solution.

Aqueous solution of CuSO_4 was observed to change in color tone according to the pH or the concentration of the solution, as will be shown in the following.

blue $\xrightarrow{\text{diluted with H}_2\text{O}}$ greenish blue $\xrightarrow{\text{alkalified with NaOH or KOH}}$ bluish blue
 $\xrightarrow{\text{alkalified with NH}_4\text{OH}}$ bluish violet

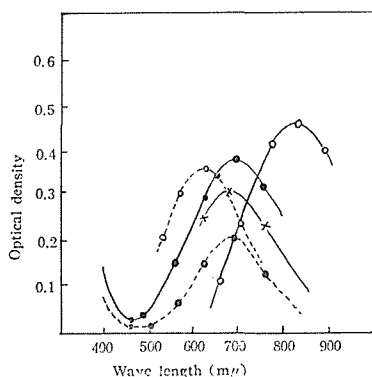


Fig. 5. Absorption spectrum of CuSO_4 in alkaline solution.

Concentration of CuSO_4 is $0.01M$. (○—○—○) Absorption curve of CuSO_4 in aqueous solution, (●—●—●) with CuSO_4 in $0.02M$ NaOH solution containing tartrate, (×—×—×) with $1.0M$ NaOH solution, (○····○····○) with NH_4OH solution, (●····●····●) pyocyanine aqueous solution.

From the further studies on the relation between color tone and absorption spectrum, it was found, as shown in Fig. 5, that aqueous solution of CuSO_4 showed absorption maximum in the region from 600 to 900 $m\mu$ according to the alkalinity of the solution, and that when its absorption maximum was exhibited at 690 $m\mu$, the solution coincided wholly with pyocyanine in color tone. And if the colors were kept in the same intensity, optical density of the solutions would be estimated at an equal value. Within a definite region, absorption maximum of CuSO_4 solution was found to move toward shorter wave length and to increase in color intensity according to increasing alkalinity of the solution (see Figs. 5 and 6).

As will be described later, the mixture of NaOH and CuSO_4 at the molar ratio of 2:1 showed an absorption maximum at $690\text{ m}\mu$. When CuSO_4 solution was made alkaline with NH_4OH , its color was observed to be bluish violet differing from that of pyocyanine.

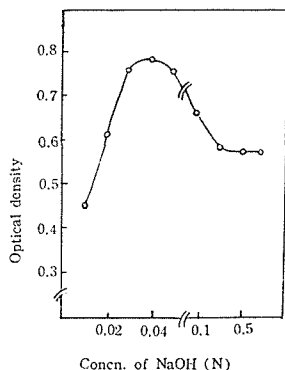


Fig. 6. Effect of concentration of NaOH on color intensity of CuSO_4 solution.

Concentration of CuSO_4 was $0.02M$. Color intensity was represented as the value of optical density at $690\text{ m}\mu$.

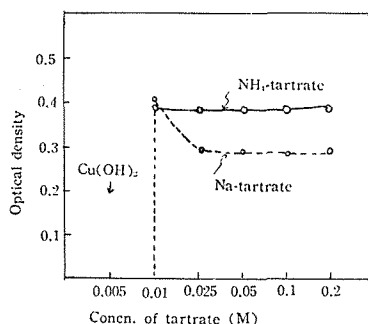


Fig. 7. Effect of concentration of tartrate on color intensity of CuSO_4 solution.

Concentration of CuSO_4 was $0.01M$ and NaOH was $0.02M$.

Preparation of standard solution with CuSO_4 . Alkaline CuSO_4 solution was observed not only to move toward shorter parts of wave length in its absorption maximum but to decrease in its color intensity, according to the increasing alkalinity of the solution. Similar phenomenon was observed with CuSO_4 solution containing excess of sodium or potassium tartrate, as will be illustrated later. However it has been found that when ammonium tartrate was used instead of sodium or potassium salt, the solution was satisfactorily stable (Fig. 7). Standard solution was prepared by the following procedure: in 50 ml of distilled water, 0.4994 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.6 g of ammonium tartrate were dissolved, and $0.08M$ NaOH solution was added so as to adjust the final volume to 100 ml. In this case, ammonium tartrate may, to some extent, be used in excessive amount but the others must not be so, because for the standard solution the following relation should be provided:

$$N/C = k$$

where N and C are the molar concentrations of NaOH and CuSO_4 , respectively. In order to show the absorption maximum of the standard solution at $690\text{ m}\mu$, a constant, k is calculated as 2.

Effect of concentration of NaOH on color intensity. As was described before, the concentration of NaOH existing in the standard solution must be controlled according to that of CuSO_4 so as to keep the molar ratio of 2:1. As shown in Fig. 6 which represents the results with $0.002M$ CuSO_4 solution, the values of optical density at $690\text{ m}\mu$ were observed to be increasing until the concentration of NaOH was increasing to $0.04M$ where the value of optical density,

i.e. the color intensity attained to the maximum and then was decreasing gradually as the concentration of NaOH was more increasing. At the concentration of NaOH higher than 0.25M, optical density was observed to be nearly constant.

Effect of sodium and potassium tartrates. In order to avoid precipitation of CuSO_4 in alkaline solution, addition of tartrate is necessary. It was found, as was mentioned before, that sodium or potassium tartrate brought a decreasing effect on color intensity of CuSO_4 solution as in the case where NaOH was used at an excessive concentration. However, as seen in Fig. 7, ammonium tartrate did not reveal any effect on the color intensity of the solution even when it was excessively used.

Test and adjustment of standard solution. With the standard solution, optical density was estimated using Beckman spectrophotometer. As shown in

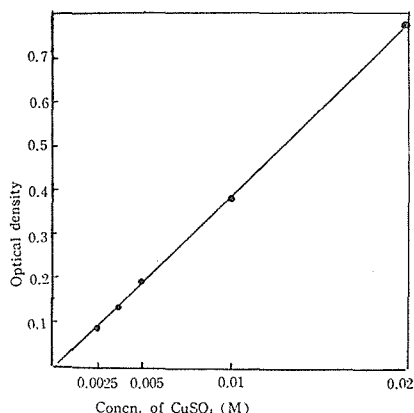


Fig. 8. Relation between color intensity and concentration of CuSO_4 in alkaline solution.

Color intensity was expressed as the value of optical density at 690 m μ where the molar concentrations of CuSO_4 and NaOH were kept at the ratio of 1:2.

Fig. 8, the relation between the value of optical density and the concentration of CuSO_4 to which the ratio of NaOH was kept constant as $k=2$, was observed to be expressed as a straight line. Table 4 shows the result of the experiment on the comparison of optical densities between the standard solution and pyocyanine which was adjusted to be macroscopically equal in color intensity to the stand-

Table 4. Comparison of optical densities between CuSO_4 and pyocyanine.

Concentration of CuSO_4	CuSO_4	Optical density of Pyocyanine
M/400	0.097	0.093
M/300	0.128	0.132
M/200	0.191	0.198
M/100	0.386	0.380
2M/100	0.770	0.776

Optical densities were observed at 690 m μ respectively, with the solutions revealing equal color intensity.

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ard solution. It is obvious from the above result that in the range of concentration shown in Table 4, the value of optical density of standard solution is directly parallel to that of pyocyanine solution. The amount of pyocyanine is calculated by the concentration of standard, as will be illustrated as follows: when the values of optical densities indicated in Table 4 were applied to 0.01M CuSO₄ solution, the arithmetic mean value was obtained as 0.385 which was found to be corresponding to 1.881mg% pyocyanine. From this value, the standard solutions were arranged so as to correspond to series of 0.001~0.008% pyocyanine solution in the test tubes of the same diameter. In order to avoid the evaporation of ammonia from the standard solution, rubber stoppers were used on each tube.

Measurement. Measurement of pyocyanine was performed as follows: pyocyanine aqueous solutions of various concentrations were taken into the test tubes mentioned above and adjusted so as to become equal in color intensity to series of the standard solution. This result will be seen in Table 5. In the

Table 5. Accuracy of estimated value of pyocyanine.

Pyocyanine known (mg%)	No. of measurement	Standard errors (mg%)	Standard deviation (mg%)	Confidence limits (mg%)
1.0	8	1.0±0.069	1.060±0.031	1.0±0.0733
2.0	8	2.0±0.095	2.080±0.047	2.0±0.1111
3.0	8	3.0±0.137	3.120±0.068	3.0±0.1608
4.0	8	4.0±0.299	4.270±0.127	4.0±0.3003
5.0	8	5.0±0.407	5.370±0.176	5.0±0.4160

Accuracy of the values obtained macroscopically were tested by using Beckman spectrophotometer. Confidence limit was concluded at the degree of freedom of 7 and perilous rate of 5%.

range shown in Table 5, pyocyanine was generally estimated at the accuracy of errors less than ±8%. Although personal errors should be unavoidable in the present method, these errors were suggested to be within the confidence limit (perilous rate is 5%; degree of freedom, 7).

Therefore, the method mentioned above is considered to be fairly satisfactory for the determination of pyocyanine in the cultured solution.

Determination of pyocyanine in cultured solution, The color shown by standard solution was adjusted in comparison with the pyocyanine aqueous solution prepared from crystal sample. When the sample solution of pyocyanine obtained from cultured solution did not coincide in color tone with the standard solution, contamination due to α -hydroxyphenazine would be taken into account. Therefore, in general, the sample is desirable to take the following procedure: the acidified aqueous solution of pyocyanine obtained after the same manner mentioned before by transference from chloroform extract of cultured solution, is washed with chloroform once or twice and then is neutralized with NH₄OH or Na₂HPO₄.

SUMMARY

1. From the cultured solution of the variant strain of *Pseudomonas aeruginosa*, pyocyanine was isolated as crystals of free form and of its various derivatives, and it was observed that the pure crystal of pyocyanine could never be obtained by merely repeating recrystallization from both water and other organic solvents, unless pyocyanine chloroplatinate was employed as a starting material.

2. It was found that pyocyanine chloroplatinate was not only easily obtained in pure state but also so stable as to be preserved for a long period without any deterioration.

3. By using Beckman model DU spectrophotometer, absorption spectrum of pyocyanine was reexamined, and the results considerably different from those of other investigators were obtained.

4. For the photometric determination using Beckman photometer, the regression line expressing the relation between the value of optical density (y) and the amount of pyocyanine (x) has been proposed as the following equations:

$$Y = 0.2047x, \text{ at } 690 \text{ m}\mu \text{ with pyocyanine aqueous solution;}$$

or
$$Y = 0.1167x, \text{ at } 520 \text{ m}\mu \text{ with pyocyanine acid aqueous solution.}$$

And the result of the determination obtained from the present equations was found to be reliable at the precision of the errors less than $\pm 3\%$.

5. For the determination of pyocyanine by the above method, the effect of α -hydroxyphenazine and other impurities in the cultured solution was found to be negligible in the measurement at $690 \text{ m}\mu$ with neutral or alkaline aqueous solution, so that it was ascertained that purification of pyocyanine would not be so much required, if its solution was completely clear.

6. In order to establish a simple colorimetric method of the determination of pyocyanine in comparison with standard solution, some photometric study has been carried out on the relation between alkalinity and color tone or intensity of CuSO_4 solution, and it was observed that when the concentrations of NaOH and CuSO_4 in the standard solution were kept at the molar ratio of 2 : 1, this solution revealed absorption maximum at $690 \text{ m}\mu$ and wholly corresponded in color tone to pyocyanine solution.

7. It was found that when NH_4 -tartrate was used for protecting Cu ion from precipitation of its hydroxide, the standard solution was very much stable in color tone or intensity, while Na - or K -tartrate could never be employed, because a decreasing effect on color intensity of the solution was brought about.

8. It was ascertained that the standard solution of $0.01M$ CuSO_4 was corresponding to $1.881 \text{ mg}\%$ pyocyanine and experimental result was found to be reliable at the precision of errors less than $\pm 8\%$.

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